Available online at www.sciencedirect.com
ScienceDirect
Virus Research 127 (2007) 26–33
Virus Research
www.elsevier.com/locate/virusres

Identification and characterization of Raspberry mottle virus, a novel member of the *Closteroviridae*

Ioannis E. Tzanetakis [a,*], Anne Halgren [a], Nola Mosier [b], Robert R. Martin [b]

[a] *Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331, United States*
[b] *Horticultural Crops Research Laboratory, USDA-ARS, Corvallis 97330, United States*

Received 17 February 2007; received in revised form 12 March 2007; accepted 13 March 2007
Available online 19 April 2007

Abstract

Raspberry mosaic is one of the most important viral diseases of raspberry. Four virus and virus-like agents, two of which are poorly characterized, have been implicated in the disease complex based on symptom development in *Rubus* indicators. Three novel viruses were identified in a red raspberry plant that caused typical raspberry mosaic symptoms when grafted onto indicators. This communication focuses on one of these viruses, Raspberry mottle virus (RMoV), a new member of the family *Closteroviridae*. The complete nucleotide sequence of RMoV has been determined and exceeds 17 kilobases encoding 10 genes. The genome organization of RMoV is similar to that of *Beet yellows virus*, the type member of the *Closterovirus* genus, and phylogenetic analysis using the polymerase conserved motifs and the heat shock protein 70 homolog revealed a close relationship of RMoV with Strawberry chlorotic fleck associated virus and *Citrus tristeza virus*, which suggests the possibility of an aphid vector. The virus was detected in symptomatic raspberry plants in production areas in mixed infections with several other viruses, indicating that RMoV may impact raspberry production.

Keywords: *Closterovirus*; *Crinivirus*; *Rubus*; dsRNA; Phylogenetic analysis; Detection

1. Introduction

More than 40 virus and virus-like agents infect *Rubus* spp. (raspberry and blackberry) worldwide (Converse, 1987b; Halgren et al., 2007; Jones et al., 2006; Martin et al., 2004; Tzanetakis and Martin, 2004), many of which cause significant yield losses (Strik and Martin, 2003; Halgren et al., 2007). Unlike viruses causing symptoms and devastating diseases, there are several that remain latent and cause symptoms only when found in complexes with other viruses and graft-transmissible agents (Jones, 1980, 1982), making their impact on yield and fruit quality difficult to determine. Detection of viruses lacking serological or nucleic acid based methods is achieved by grafting suspect material onto indicator plants.

The USDA-ARS Horticultural Crops Research Laboratory (HCRL) in Corvallis, Oregon, maintains a collection of *Rubus* accessions for breeding purposes, and graft indexing is performed routinely on these plants for identification of pathogens. One symptomless red raspberry accession, 'Glen Clova' #8 (GC-8), that originated in Scotland and is maintained in an insect-free screenhouse, caused severe symptoms including leaf mottling, epinasty and apical necrosis when grafted onto black raspberry (*R. occidentalis*) 'Munger' indicators demonstrating it was infected with one or more components of the raspberry mosaic disease (RMD). RMD was first recognized in the 1920's and is likely caused by various combinations of four or more viruses including *Black raspberry necrosis virus*, *Rubus yellow net virus*, Raspberry leaf mottle virus (RLMV) and Raspberry leaf spot virus (RLSV) (Converse, 1987b), with the latter two being uncharacterized at the molecular level. Double-stranded RNA (dsRNA) was extracted, reverse transcribed, cloned and sequenced, which resulted in the identification of three novel viruses (Martin and Tzanetakis, 2005). One of the new viruses, hereafter named Raspberry mottle virus (RMoV), is presented in this communication.

While the study with isolate GC-8 was in process, unusual mottling symptoms were observed on plants in a red raspberry plantation in northern Washington. Using the techniques employed in the GC-8 virus characterization, we found that these plants were also infected with RMoV, evidence of the

* Corresponding author. Tel.: +1 541 7384041; fax: +1 541 7384025.
E-mail address: yannis@orst.edu (I.E. Tzanetakis).

0168-1702/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2007.03.010

distribution of the virus in both North America and Europe. This finding led to the detailed study of RMoV including the complete nucleotide sequence of the GC-8 isolate. Phylogenetic analysis showed that the virus is related to members of the genus *Closterovirus*, the aphid-borne members of the family *Closteroviridae*. The variability of the virus was studied using isolates from North America and Europe and oligonucleotide primers that can be used for detection of the virus in field grown plants were developed.

2. Materials and methods

2.1. Plant material

The GC-8 plant used in the majority of the experiments described was obtained from the Scottish Crops Research Institute (SCRI) as breeding material and maintained in an insect-proof screenhouse in Corvallis, Ore. A yellow raspberry breeding accession from the raspberry breeding program in Washington (WSU-991) was sent to our laboratory for virus indexing by Dr. P. Moore (Puyallup, Wash.). This plant caused mottling symptoms on the indicator 'Munger' after grafting. A plant of 'Meeker' grown in northern Washington (WA) exhibiting mottling symptoms was chosen among the many plants with identical symptoms for further analysis. Finally, dried tissue from RLMV, RLSV and Raspberry vein chlorosis virus (RVCV)-infected plants, obtained under permit from SCRI, was assayed for the presence of the virus to test the possibility that RMoV is one of the previously described graft-transmissible agents of *Rubus* (Converse, 1987b).

2.2. Host range and transmission studies

Grafting was performed as described previously (Converse, 1987a). For mechanical inoculations, young, fully developed leaves from the GC-8 plant were collected and ground in phosphate buffered saline (PBS), pH 7.4, containing 2% nicotine, at wt/vol ratio of 1:20. Five plants of *Chenopodium quinoa, Cucumis sativus, Nicotiana benthamiana, N. occidentalis, N. tabacum and Tetragonia tetragonioides* were mechanically inoculated after being dusted with carborundum (600 mesh) to facilitate entry of the virus into cells. All herbaceous indicator plants were tested by reverse transcription-polymerase chain reaction (RT-PCR) approximately one month after inoculation.

2.3. Nucleic acids isolation

Total RNA was extracted by a combination of the Halgren et al. (2007) and Rott and Jelkmann (2001) procedures. Briefly, tissue was homogenized and processed through the isopropanaol precipitation step as described (Halgren et al., 2007). The precipitated RNA was resuspended in 500 μl of wash buffer and 25 μl of glass milk (Rott and Jelkmann, 2001). The glass milk was pelleted by pulse centrifugation to 6000 rpm and washed with 500 μl of wash buffer. After a brief centrifugation, glass milk was dried in a speed vac and the RNA was resuspended in 150 μl TE. Double-stranded RNA was extracted as described (Tzanetakis et al., 2005a).

Table 1
List of the oligonucleotide primers used for detection, amplification of the genome and genetic variability studies of Raspberry mottle virus

Primer name	Nucleotide sequence (5′–3′)
Detection	
CPhF	CGAAACTTYTACGGGGAAC
CPhR	CCTTTGAAYTCTTTAACATCGT
CPF	GTAAGGAGATATGGCGGA
CPR	CAGTATGGCAGCCTCTTG
Genomic variability	
HSPA	CTCTACTAACGCGATCTG
HSPB	CATAGTACCTTGAGGAGC
CPA	ACACCGAAGCTGTTCGTT
CPB	AATACACTCAAGCGGAGC
Genome amplification	
AP	GGCCACGCGTCGACTAGTAC$(T)_{18}$
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
5′ R	GTTAGGGTGCAGGGGTTTCA
RC	CGGAGGATTTCGGGGTCGGTGGCGA
Beg R	GAGGGAATCGGAGAGAGGTGAGAAATC
5′ F	TGAAACCCCTGCACCCTAAC
Pol 2500 R	GCGAACTTGTGTAGAAGAGCATTC
Pol beg F	AGGTTTCGTATTGGGCGCG
Pol mid R	GAGACGAGCCCAGAGCAGAGATCGAGAAG
Pol mid F	CTTCTCGATCTCTGCTCTGGGCTCGTCTC
Pol end R	CGATGAATATAGTGAATTTGACGAC
Pol end F	GTCGTCAAATTCACTATATTCATCG
Mid R	CAAGAAACTAACATCGACGAAGTGA
Mid F	TCACTTCGTCGATGTTAGTTTCTTG
HSP beg R	GACGGATGTAGTTTAGCACGG
HSP beg F	GTGCTAAACTACATCCGTCT
CPh R	GTGAGCGGGAGTGGA
CPh F	TGTACTGTTCCGATGAGATCTTC
p20 R	ACGAAAGTGCTACCATCAGTAAGGAGG
p20 beg F	CTATTTTATCCGTGAAAGTTGCTAC
End F	CATGGACATTGAACGAGCGGACCAGC

2.4. Detection

The reverse transcription reactions was performed as described (Halgren et al., 2007), primed with either random or specific oligonucleotide primers. Primers CPhF/CPhR and CPF/CPR (Table 1) were developed and used to amplify a 452 base fragment of the coat protein homolog (CPh) and a 514 base fragment of the major coat protein (CP) genes, respectively. The PCR program consisted of a denaturation step of 3 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, concluding with a step of 10 min at 72 °C, using Taq polymerase (GenScript, NJ) according to manufacturer's instructions, and primers at 1 or 0.5 μM each (CPh and CP, respectively).

2.5. Cloning, sequencing and genome analysis

The complete nucleotide sequence of RMoV was obtained from the GC-8 accession and the methodology used was essentially identical to that used for another closterovirus, Mint

virus-1 (Tzanetakis et al., 2005a; Tzanetakis et al., 2005c). RMoV-specific sequences were identified using blastn and blastx to compare with sequences found in the GenBank databases (Altschul et al., 1997). Oligonucleotide primers were developed from the RMoV sequences and used for RT-PCR amplification of the genome (Table 1). The 5′ and 3′ termini of the genome were acquired by 5′ and 3′ RACE using primers AAP and AP (Table 1; Invitrogen) as described previously (Tzanetakis and Martin, 2004). Consensus of the complete genome and all other sequences presented were acquired utilizing CAP3 (Huang and Madan, 1999) and were built using the clones from shotgun cloning where applicable, at least four clones of the RT-PCR amplicons and direct sequencing of the RT-PCR products, for an at least 5× genome coverage. The genome of RMoV has been deposited in Genbank and given the accession number DQ357218. The genetic diversity of the virus was examined by sequencing the heat shock protein 70 homolog (HSP70h; accession numbers DQ016611–EF114208) and CP genes (accession numbers DQ016612–EF114209) of the WA and RLM isolates, respectively.

The open reading frames (ORF) were identified using the ORF finder software at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Conserved protein domains were identified with CDD (Marchler-Bauer et al., 2003) and the exact amino acid (aa) regions were recognized after alignment of known closteroviral motifs using ClustalW (Thompson et al., 1994). ClustalW was also applied for phylogenetic analysis (neighbor-joining algorithm, Kimura's correction and bootstrap analysis consisting of 1000 pseudoreplicates). Trees were viewed on TreeView (Page, 1996). The putative transmembrane domains of the proteins were predicted with TMHMM (Krogh et al., 2001) while RNA secondary structure was predicted using Mfold (Zuker, 2003).

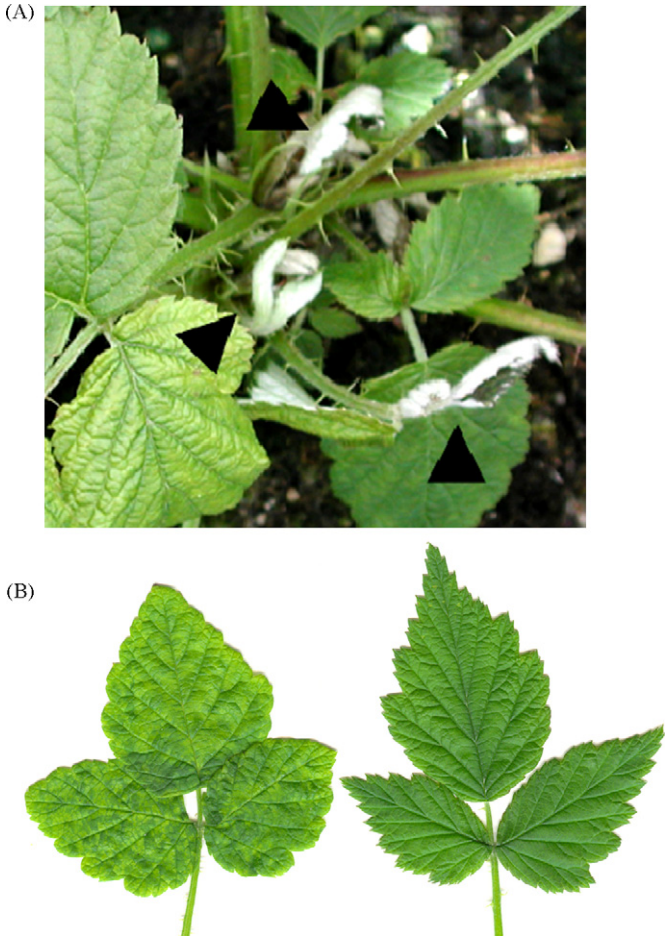

Fig. 1. (A) Symptoms of 'Munger' indicators grafted with 'Glen Clova' 8 tissue. Arrows point to the necrosis of the apical meristem. (B) Leaf symptoms of 'Munger' indicators grafted with the Raspberry mottle virus 'WSU 991' isolate. Left: Leaf from grafted plant showing mottling symptoms; right: leaf from 'Munger' seedling.

3. Results

Severe symptoms developed on 'Munger' black raspberry indicators about 3 weeks after grafting with GC-8 leaves. Symptoms included mottling, severe stunting and apical necrosis (Fig. 1A). Mottling developed on 'Munger' after grafting with WSU-991, free of the other viruses found in GC-8 (Fig. 1B) and for this reason we propose the name Raspberry mottle for the new virus. RMoV was not mechanically transmitted to the range of herbaceous indicators used. Multiple dsRNA bands were obtained from GC-8 and WSU-991, unlike healthy 'Munger' seedlings where no dsRNA was obtained (data not shown). The pattern of the dsRNA was similar to that obtained from closteroviruses (Tzanetakis et al., 2005b; Tzanetakis et al., 2005c), with a high MW band of about 17 kilobases, and several lower MW bands, with a prominent 1.2 kilobase band, probably corresponding to virus subgenomic or defective RNAs (Fig. 2). The dsRNA was used as a template for cloning, and about 11 kilobases of the RMoV genome were obtained using the shotgun approach as determined by comparison to closterovirus sequences found in GenBank (Altschul et al., 1997).

The complete nucleotide sequence of the GC-8 isolate of RMoV has been determined and consists of 17,481 nucleotides (nt) (G + C content: 47.5%) encoding 10 putative ORFs (Fig. 3). The first AUG codon of the GC-8 isolate is found between nt 340–342. The 1a polyprotein is 2819 aa and approximately 313 kDa.

The N′ terminus of 1a protein contains motifs of papain-like proteases. Sequence comparisons indicated that there are probably two papain-like leader proteases (L1-Pro and L2-Pro, respectively), similar to those of Strawberry chlorotic fleck associated virus (SCFaV) (Tzanetakis and Martin, 2007), *Citrus tristeza virus* (CTV) (Karasev et al., 1995) and *Grapevine leafroll associated virus-2* (Meng et al., 2005). The two domains are 477 and 214 aa, respectively, and both cleavage sites are predicted to be between Gly residues. The conserved His and Cys residues of the closterovirus proteases (Peng et al., 2001) are found in positions 399, 460 for L1-Pro and 129, 191 for L2-Pro, respectively. The autocleavage of the two proteases releases a 236 kDa protein with signature methyltransferase motifs at the N-terminus and RNA helicase motifs at the C-terminus. The methyltransferase motifs (Rozanov et al., 1992) are predicted between residues 776 and 1072 of 1a while the helicase motifs are found between 2429 and 2698 (Candresse et al., 1990).

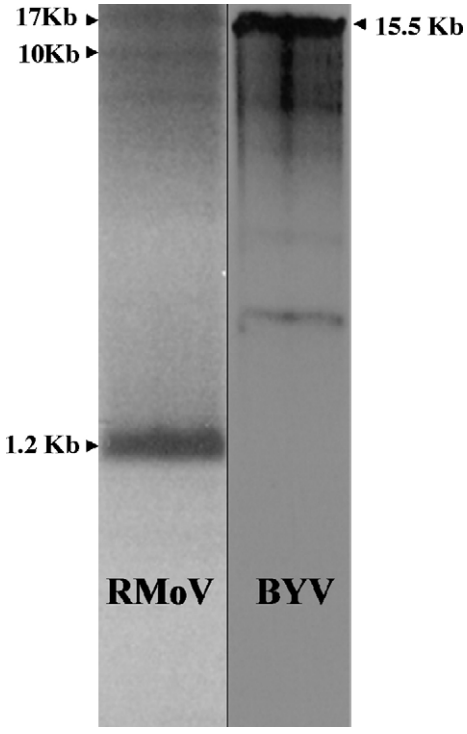

Fig. 2. Double-stranded RNA (dsRNA) extracted from Raspberry mottle virus (RMoV) infected material. Left: dsRNA obtained from of RMoV infected plant, free of known *Rubus* viruses. Arrowheads point to a predominant 1.2 kilobases and a ~ 17 kilobases band; right: dsRNA pattern of *Beet yellows virus* used as standard.

Both domains show greater similarity to orthologous domains of SCFaV with 52 and 58% aa identity for the methyltransferase and helicase domains, respectively. The region between the methyltransferase and helicase contains four putative transmembrane domains (aa 1789–1812, 1824–1846, 1856–1878, 2050–2072; Krogh et al., 2001). The virus RNA dependent RNA polymerase (RdRp, 1b) is probably expressed by a + 1 ribosomal frameshift, as is the case with other members of the family (Martelli et al., 2002) although the surrounding region does not have any slippery sequences nor structures that could facilitate the frameshift (Zuker, 2003). It is possible that the frameshift is facilitated by a newly identified antisense mechanism (Henderson et al., 2006). The 54 kDa polymerase domain is 467 aa and has the eight conserved motifs of the RNA dependent RNA polymerases identified by Koonin (1991) between residues 108 and 346. The region shows the highest aa identity with orthologous domains of other members of the *Closterovirus* genus, exceeding 67% identity with all sequenced members of the group and reaching almost 80% for some isolates of CTV. There is a 645 nt intergenic region between the RdRp and a small ORF (ORF 2), which is located between nt 10846–11031 encoding a 7 kDa protein. This 7 kDa protein contains a transmembrane domain between residues 31 and 53 (Krogh et al., 2001). A similar ORF (ORF 3) is found between nt 11082–11237 encoding a 6 kDa hydrophobic protein with a transmembrane domain found between residues 11 and 33. A Cys in position 3 indicates that the peptide probably has a similar function to the small hydrophobic peptide of *Beet yellows virus* (BYV). BYV p6 is one of the five proteins involved in virus movement. The Cys at position 3 is involved in disulfide bridge formation and dimerization of the protein, a property essential in virus movement (Peremyslov et al., 2004). ORF 4 (nt 11244–13043) encodes the HSP70h of the virus, the hallmark gene of all closteroviruses. The 65 kDa protein is involved in virus movement and virion assembly (Dolja et al., 2006) and has the five conserved ATPase motifs of HSP70s between residues 3 and 336 (Bork et al., 1992) probably involved in virus movement. The RMoV HSP70h is related most closely to the orthologous gene of SCFaV with more than 50% aa sequence identity. ORF 5 (nt 12943–14478) encodes a putative 58 kDa protein. Sequence analysis indicated the protein is similar to closterovirus CPh (Napuli et al., 2003), another of the movement proteins of the virus (Dolja et al., 2006). The protein has about 40% aa sequence identity with the orthologous proteins of CTV and SCFaV. The conserved Arg and Asp residues involved in stabilization of the virion structure (Dolja et al., 1991; Jagadish et al., 1993) are found in positions 412 and 451, respectively. ORF 6 and 7 (nt 14555–15247 and 15372–15968) encode the two coat proteins of the virus: the 25 kDa minor coat protein (CPm) and the 22 kDa CP, respectively. The proteins encapsidate the genome and are also involved in virus movement (Dolja et al., 2006). The proteins are most closely related to orthologous proteins of CTV with about 40% aa sequence identity residues. The conserved

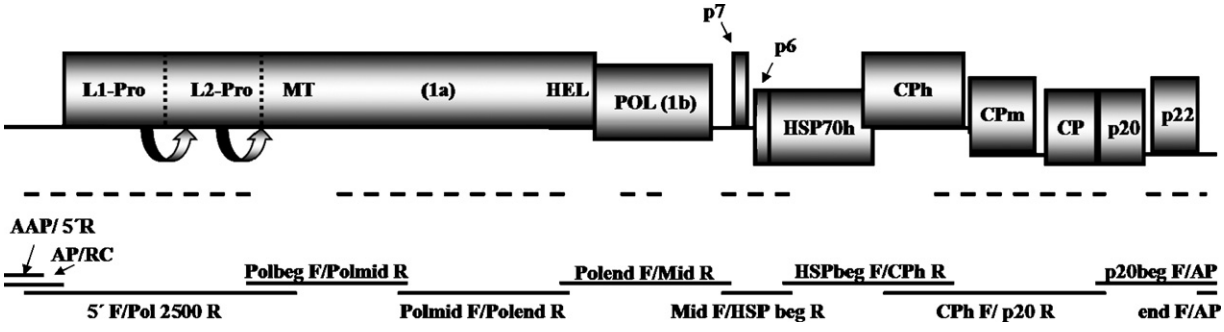

Fig. 3. Schematic representation of the genome organization of Raspberry mottle virus. Abbreviations: L1-Pro: leader papain-like protease 1; L2-Pro: leader papain-like protease 2; MT: methyltransferase; Hel: helicase; Pol: RNA-dependent RNA polymerase; HSP70h: heat shock protein 70 homolog; CPh: coat protein homolog; CPm: minor coat protein; CP: major coat protein. Dashed bars represent regions of the virus acquired by shotgun cloning of double-stranded RNA. Solid bars represent the reverse transcription-polymerase chain reaction products used to acquire the genome of the virus. Primers are listed in table 1. Open reading frames are not to scale.

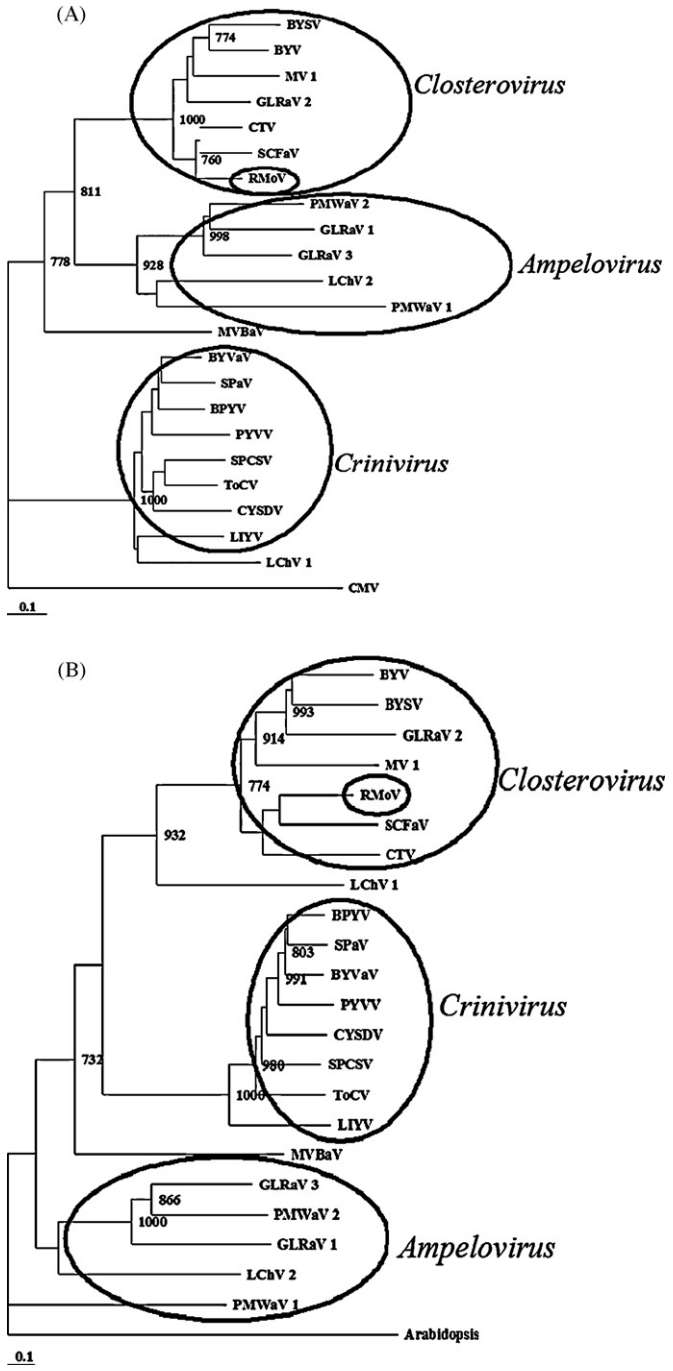

Fig. 4. (A) Phylogram of the polymerase conserved motifs of Raspberry mottle virus (RMoV) and other closteroviruses. Abbreviations and GenBank accession numbers: BPYV, *Beet pseudo yellows virus*, NP940796; BYV, *Beet yellows virus*, NP733949; BYSV, *Beet yellow stunt virus*, AAC55659; BYVaV, Blackberry yellow vein associated virus, AAV40963; CTV, *Citrus tristeza virus*, NP733947; CMV, *Cucumber mosaic virus*, NP049324; CYSDV, *Cucurbit yellow stunting disorder virus*, AAM73639; GLRaV 1, *Grapevine leafroll associated virus 1*, AAF22738; GLRaV 2, *Grapevine leafroll associated virus 2,* AAC40856; GLRaV 3, *Grapevine leafroll associated virus 3*, AAC40705; LIYV, *Lettuce infectious yellows virus*, AAA61798; *Little cherry virus 1*, NP733945; LChV 2, *Little cherry virus 2*, AAP87784; MVBaV, Mint vein banding associated virus, AAS57939; MV 1, Mint virus 1, AAW32893; PMWaV 1, *Pineapple mealybug wilt associated virus 1*, AAL66709; PMWaV 2, *Pineapple mealybug wilt associated virus 2*, AAG13939; PYVV, *Potato yellow vein virus*, CAD89680; RMoV, Raspberry mottle virus, DQ357218; SCFaV, Strawberry chlorotic fleck associated virus, DQ860839; SPaV, Strawberry pallidosis associated virus, AY488137; SPCSV, *Sweet potato chlorotic stunt virus*, NP733939; ToCV, *Tomato chlorosis virus*, AAY21795. CMV is used as the outgroup. (B) Phylogram of heat shock protein 70 homolog of RMoV and other closteroviruses. GenBank accession numbers: Arabidopsis, *Arabidopsis thaliana* putative heat shock protein 70, AAN71949; BPYV, AAQ97386; BYSV, AAC55662; BYV, NP041872; BYVaV, AAV40966; CTV, NP042864; CYSDV, NP851572; GLRaV 1, AAF22740; GLRaV 2, AAR21242; GLRaV 3, NP813799; LIYV, NP619695; LChV 1, NP045004; LChV 2, AF531505; MVBaV, AAS57941; MV 1, AAW32895; PMWaV 1, AAL66711; PMWaV 2, AAG13941; PYVV, CAD89682; RMoV, DQ357218; SCFaV, DQ860839, SPaV, AAO92347; SPCSV, NP689401; ToCV, AF024630. The Arabidopsis protein is used as the outgroup. Bootstrap values are shown as percentage value and only the nodes over 70% are labeled. The bars represent 0.1 amino acid changes per site.

Arg and Asp residues are found in positions 142 and 183 for the CPm and 111 and 153 for the CP. ORF 8 (nt 16026–16556) encodes a putative 20 kDa protein. BLAST searches failed to reveal any similarity of the protein with other plant virus proteins and its function remains unknown. The final ORF (nt 16628–17248) encodes a 23 kDa protein, an ortholog of an RNA silencing suppressor of BYV (Reed et al., 2003; Lu et al., 2004). This putative protein of RMoV contains the motif identified previously at the C′ terminus in orthologous proteins (Tzanetakis et al., 2005c). The protein has about 25% aa sequence identity with the orthologous protein of CTV and SCFaV.

The 3′ terminus is 233 nt and contains an adenosine rich region near the end, also found in other members of the family (Tzanetakis et al., 2005c), with unrecognized function. Sequence and secondary structure analysis failed to identify any sequences that could facilitate a pseudoknot formation similar to those predicted in *Potato yellow vein virus* and SCFaV.

Phylogenetic analysis using two regions of the closteroviral genome, the polymerase and the HSP70h, reveals clustering of RMoV with members of the *Closterovirus* genus as predicted from sequence analysis (Fig. 4)

A primer set developed against the HSP70h of GC-8 isolate failed to detect the virus in any of WA plants that exhibited leaf mottling. These plants were tested for *Raspberry bushy dwarf virus, Black raspberry necrosis virus*, Strawberry necrotic shock virus and Blackberry yellow vein associated virus (BYVaV) but non of these viruses was consistently found in these plants indicating the symptoms were caused by a combination of viruses or a novel agent. DsRNA was extracted and cloned as described for GC-8 from a WA plant that exhibited leaf mottling and a portion of the HSP70h gene was obtained. The nt sequence identity of an approximate 500 base region of the gene from the GC-8 and WA plants was only 79%, while the aa identity exceeded 90% indicating that these plants were infected with RMoV.

In order to study the sequence diversity observed in portions of the HSP70h and develop optimal detection protocols, the HSP70h and the CP from the WA and the RLM isolates were obtained. The WA HSP70h is 598 aa, in contrast to the 599 aa of the GC-8 isolate. The open reading frame (ORF) has 79% nt identity between isolates, while they share 92 and 96% aa sequence identity and similarity, respectively. The CP ORF is 198 aa with over 97% nt and 98% aa sequence identity between isolates. In contrast the two genes of RLM show great similar-

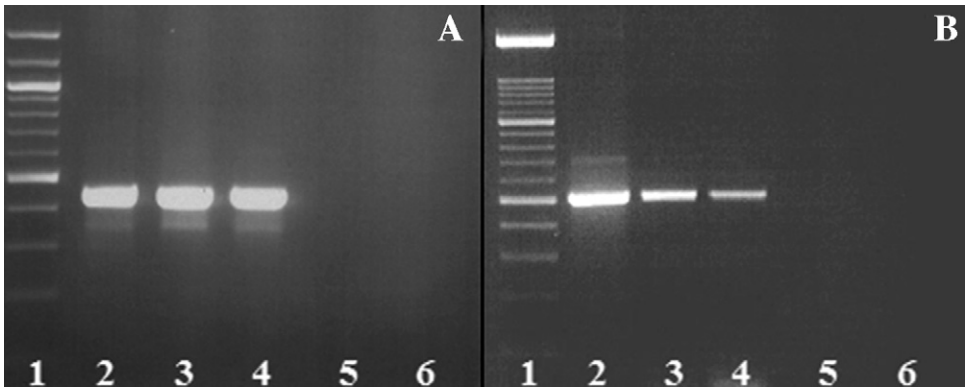

Fig. 5. Detection of Raspberry mottle virus (RMoV) using reverse transcription-polymerase chain reaction. (A) Amplification of a 452 base region of the coat protein homolog gene. Lane 1: 100 base pair marker; lanes 2–4: RMoV infected red raspberry from Scotland, red raspberry from Washington, and yellow raspberry from Washington, respectively; lane 5–6: healthy red and black raspberry seedlings. (B) Amplification of a 514 base region of the major coat protein gene. Lane 1: 100 base pair marker; lanes 2–4: RMoV infected red raspberry from Scotland, red raspberry from Washington, and yellow raspberry from Washington, respectively; lane 5–6: healthy red and black raspberry seedlings.

ity to the GC-8 isolate with 97% nt sequence identity for the HSP70h and 96% for the CP. The aa sequences are 97 and 99% identical to the orthologous genes of GC-8. Shotgun cloning from dsRNA obtained from the WSU-991 isolate showed that the two isolates share 97–98% nucleotide identity in regions of 1a, the HSP70h and the CP (Tzanetakis, unpublished data), suggesting that the two isolates are very similar.

Because of the problems with detection of the WA isolate, several sets of oligonucleotide primers were tested for detection of RMoV. The sets presented were the two that detected all six isolates (GC-8, WSU-991, WA, RLM, RLS and RVC) in addition to isolates from black and red raspberry from the Pacific Northwest (data not shown). Validation of the tests was done by sequencing at least five of the amplicons from each primer set. They were all determined to be RMoV-specific.

More than 20 plants from northern Washington with symptoms similar to that of the WA isolate were assayed for the presence of RMoV with both detection primer sets. While the majority were found infected with RMoV, some were not found infected with the virus.

4. Discussion

Graft-transmissible agents have been associated with RMD for more than 50 years, but only two of them are characterized at the molecular level (Jones et al., 2002; Halgren et al., 2007). Grafting of GC-8 onto indicators caused symptoms typical of RMD and this prompted us to identify which of the RMD agents were infecting this plant. Three new viruses were identified (Martin and Tzanetakis, 2005), indicating that RMD may be caused by previously unrecognized viruses or a complex of the new and the old viruses. This communication focuses on one of the viruses, a novel member of the *Closteroviridae* named RMoV.

The close relationship of the virus with members of the *Closterovirus* genus, the only members of the family that are potentially mechanically transmitted (Martelli et al., 2002), led to the investigation of that property. RMoV was not found to be mechanically transmitted to the range of herbaceous indicators used. This could be a property of the virus or a combination of the inefficient mechanical transmission of most closteroviruses with the inhibitors found in *Rubus* and related species (Fulton, 1966).

The low titer of closteroviruses (Liu et al., 2000; El Beaino et al., 2005; Tzanetakis et al., 2005c) makes detection a challenging undertaking. Because of that property, RT-PCR is the preferred detection method for closteroviruses. The disadvantage of the method is the potential of false negatives because of mismatches between primers and template that can occur in isolates as diverse as those found with RMoV. In order to minimize that possibility, two sets of primers for two genomic regions of the virus were developed for routine detection (Fig. 5).

The diversity of closteroviruses is well established (Karasev, 2000; D'Urso et al., 2003). The extreme nt diversity of the HSP70h ORF for isolates of the same species, has also been found in other closteroviruses (Tairo et al., 2005; Ruiz-Ruiz et al., 2006). The contrast between the diverse HSP70h ORF (79% nt identity) to the very similar CP ORF (97% nt identity) is a feature found in other closteroviruses and especially CTV, where sequence diversity between isolates decreases from the 5′ to 3′ end of the genome (Mawassi et al., 1996). The different size of the HSP70h is unusual, although there are reports of ORFs of considerably different sizes in closteroviruses (Tzanetakis and Martin, 2004). The consensus sequence of the terminal region of RMoV HSP70h was obtained after alignment of six clones of the PCR products, all of which were identical.

An interesting feature of the GC-8 isolate was observed when trying to obtain the 5′ terminal sequence using dsRNA as template. In the first 300 nucleotides of the genome there were two gaps that resulted in about 70 nt missing compared to the homologous region of the WSU-991 and the RLM isolates (accession numbers EF421975-EF421976, respectively).

This region of the GC-8 isolate was sequenced at least 15 times, from clones of PCR amplicons derived from three individual RT and four PCR reactions. In all cases the gaps were present. The region is not highly structured (Zuker, 2003), nor GC rich (G + C content: 47.5%). We used methyl mercury hydroxide in the denaturation step of the dsRNA and the RT was performed at

60 °C, minimizing the possibility of the gap regions being inaccessible during the RT reaction. With the gaps the first AUG of the genome was at position 1314. A 5′ UTR of that size in unprecedented and would give no obvious advantage to the virus. We tried to obtain the region using single-stranded RNA as template and the gaps in the sequence were absent placing the start codon of 1a at position 340–342. This finding is perplexing, but highlights the importance of closely examining anomalies observed in sequences of new viruses to ensure they are not the result of difficulties with template.

The presence of two small hydrophobic proteins in the genome of RMoV and the area of about 650 nt between the polymerase and p7 may provide significant insight in the evolution of the *Closteroviridae*. The transmembrane domain of p7 shows 37% similarity to the transmembrane domains of CTV p33 and SCFaV p28, indicating a possible distant relationships between the proteins. The large non-coding region between the polymerase and p7 may be the remains of a larger gene that was found in that position that was abolished recently or gave rise to p7. This hypothesis is reinforced by the fact that the region lacks a start codon and has a single stop codon in one of the reading frames. Che et al. (2003) found that a class of defective RNAs of CTV are similar to the genomic RNAs of criniviruses. All sequenced members of the *Crinivirus* genus have a small hydrophobic protein at the end of RNA 1 and beginning of RNA 2 with the exception of BYVaV that has the two small hydrophobic proteins at the beginning of RNA 2 (Tzanetakis et al., 2006) and *Lettuce infectious yellows virus* that lacks the first protein (Klaassen et al., 1995). The data presented here together with the findings on the defective RNAs of CTV (Che et al., 2003), and a monopartite closterovirus (Mint vein banding associated virus, Tzanetakis et al., 2005b) that shares similarities with all genera of the family, strengthens the argument that criniviruses evolved from a monopartite virus similar to members of the *Closterovirus* genus.

The northern Washington plants with mottling symptoms were not universally infected with RMoV, which suggests that RMoV is not the sole causal agent of the symptomatology but it induces symptoms when found in association with other agents. RMoV is probably widespread, as it was identified in plants from both Europe and the United States. The diversity between the GC-8 and WA isolates is not indicative of strain distribution due to geographic isolation, since WSU-991 was closely related to GC-8 even though it originated in an area proximal to that of the WA isolate. The variability may be the result of genetic bottlenecks caused by vector transmissions or host passages, similar to what has been observed for CTV (Ayllon et al., 2006; Sentandreu et al., 2006).

At this time it can not be ruled out that RMoV is the causal agent of one of the graft-transmissable diseases described for *Rubus* spp., although RMoV symptoms on grafted indicators do not correlate with those described for the graft-transmissable diseases of *Rubus* spp. (Converse, 1987b). The plants that are infected with the reference isolates of RLMV, RLSV and RVCV from SCRI were infected with RMoV, making even more complex the correlation of symptoms observed in these plants and the agents present. Our observations suggest that several of the symptoms described for many of the small fruit viruses and attributed to severe strains of known viruses probably result from mixed infections of known and unknown viruses. RMoV may be part of a complex of viruses that cause the symptoms described and associated with one or several of the *Rubus* graft-transmissible diseases.

Acknowledgments

The authors would like to thank Ruth Price for optimizing the RNA extraction protocol. We also thank Alexander Karasev and Valerian Dolja for helpful discussions and Hugh Barker and Stuart McFarland for providing us with the SCRI Raspberry leaf mottle, Raspberry leaf spot and Raspberry vein chlorosis tissue. The project was funded by the United States Department of Agriculture.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST, a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Ayllon, M.A., Rubio, L., Sentandreu, V., Moya, A., Guerri, J., Moreno, P., 2006. Variations in two gene sequences of *Citrus tristeza virus* after host passage. Virus Genes 32, 119–128.

Bork, P., Sander, C., Valencia, A., 1992. An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. Proc. Natl. Acad. Sci. USA 89, 7290–7294.

Candresse, T., Morch, M.D., Dunez, J., 1990. Multiple alignment and hierarchical clustering of conserved amino acid sequences in the replication associated proteins of plant RNA viruses. Res. Virol. 141, 315–329.

Che, X., Dawson, W.O., Bar-Joseph, M., 2003. Defective RNAs of *Citrus tristeza virus* analogous to *Crinivirus* genomic RNAs. Virology 310, 298–309.

Converse, R.H., 1987a. Detection and elimination of virus and virus-like diseases in strawberry, in: Converse, R.H. (Ed.), Virus Diseases of Small Fruits. USDA ARS Agriculture Handbook No 631, Washington, D.C., pp. 3–5.

Converse, R.H., 1987b. Virus and virus-like diseases of *Rubus* (raspberry and blackberry), in: Converse, R.H. (Ed.), Virus Diseases of Small Fruits. USDA ARS Agriculture Handbook No 631, Washington, D.C., pp. 168–253.

Dolja, V.V., Boyko, V.P., Agranovsky, A.A., Koonin, E.V., 1991. Phylogeny of capsid proteins of rod-shaped and filamentous RNA plant viruses, two families with distinct patterns of sequence and probably structure conservation. Virology 184, 79–86.

Dolja, V.V., Kreuze, J.F., Valkonen, J.P., 2006. Comparative and functional genomics of closteroviruses. Virus Res. 117, 38–51.

D'Urso, F., Sambade, A., Moya, A., Guerra, J., Moreno, P., 2003. Variation of haplotype distributions of two genomic regions of Citrus tristeza virus populations from eastern Spain. Mol. Ecol. 12, 517–526.

El Beaino, T., Saponari, M., Minafra, A., Castellano, M.A., Savino, V., Martelli, G.P., 2005. Further characterization of olive leaf yellowing-associated virus. J. Plant Path. 87, 223–228.

Fulton, R.W., 1966. Mechanical transmission of viruses of woody plants. Ann. Rev. Phytopathol. 4, 79–98.

Halgren, A.B., Tzanetakis, I.E., Martin, R.R., 2007. Characterization and detection of a virus associated with decline symptoms in black raspberry. Phytopathology 97, 44–50.

Henderson, C.M., Anderson, C.B., Howard, M.T., 2006. Antisense-induced ribosomal frameshifting. Nucleic Acids Res. 34, 4302–4310.

Huang, X., Madan, A., 1999. CAP3: A DNA sequence assembly program. Genome Res. 9, 868–877.

Jagadish, M.N., Huang, D., Ward, C.W., 1993. Site-directed mutagenesis of a potyvirus coat protein and its assembly in *Escherichia coli*. J. Gen. Virol. 74, 893–896.

Jones, A.T., 1980. Some effects of latent virus infection in red raspberry. Acta Hort. 95, 63–70.

Jones, A.T., 1982. Distinctions between three aphid-borne latent viruses of raspberry. Acta Hort. 129, 41–48.

Jones, A.T., McGavin, W.J., Geering, A.D.W., Lockhart, B.E.L., 2002. Identification of *Rubus yellow net virus* as a distinct badnavirus and its detection by PCR in Rubus species and in aphids. Ann. Appl. Biol. 141, 1–10.

Jones, A.T., McGavin, W.J., Gepp, V., Zimmerman, M.T., Scott, S.W., 2006. Purification and properties of Blackberry chlorotic ringspot, an virus species in subgroup 1 of the genus *Ilarvirus* found naturally infecting blackberry in the UK. Ann. Appl. Biol. 149, 125–135.

Karasev, A.V., 2000. Genetic diversity and evolution of closteroviruses. Ann. Rev. Phytopathol. 38, 293–324.

Karasev, A.V., Boyko, V.P., Gowda, S., Nikolaeva, O.V., Hilf, M.E., Koonin, E.V., Niblett, C.L., Cline, K., Gumpf, D.J., Lee, R.F., Garnsey, S.M., Lewandowski, D.J., Dawson, W.O., 1995. Complete sequence of the Citrus tristeza virus RNA genome. Virology 208, 511–520.

Klaassen, V.A., Boeshore, M.L., Koonin, E.V., Tian, T., Falk, B.W., 1995. Genome structure and phylogenetic analysis of lettuce infectious yellows virus, a whitefly-transmitted, bipartite closterovirus. Virology 208, 99–110.

Koonin, E.V., 1991. The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J. Gen. Virol. 72, 2197–2206.

Krogh, A., Larsson, B., von Heijne, B., Sonnhammer, E.L.L., 2001. Predicting transmembrane protein topology with a hidden Markov model. Application to complete genomes. J. Mol. Biol. 305, 567–580.

Liu, H.-Y., Wisler, G.C., Duffus, J.E., 2000. Particle lengths of whitefly-transmitted criniviruses. Plant Dis. 84, 803–805.

Lu, R., Folimonov, A., Shintaku, M., Li, W.X., Falk, B.W., Dawson, W.O., Ding, S.W., 2004. Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. Proc. Natl. Acad. Sci. USA 101, 15742–15747.

Marchler-Bauer, A., Anderson, J.B., DeWeese-Scott, C., Fedorova, N.D., Geer, L.Y., He, S., Hurwitz, D.I., Jackson, J.D., Jacobs, A.R., Lanczycki, C.J., Liebert, C.A., Liu, C., Madej, T., Marchler, G.H., Mazumder, R., Nikolskaya, A.N., Panchenko, A.R., Rao, B.S., Shoemaker, B.A., Simonyan, V., Song, J.S., Thiessen, P.A., Vasudevan, S., Wang, Y., Yamashita, R.A., Yin, J.J., Bryant, S.H., 2003. CDD, a curated Entrez database of conserved domain alignments. Nucleic Acids Res. 31, 383–387.

Martelli, G.P., Agranovsky, A.A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R.H., Dolja, V.V., Falk, B.W., Gonsalves, D., Jelkmann, W., Karasev, A.V., Minafra, A., Namba, S., Vetten, H.J., Wisler, G.C., Yoshikawa, N., 2002. The family *Closteroviridae* revised. Arch. Virol. 147, 2039–2044.

Meng, B., Li, C., Goszczynski, D.E., Gonsalves, D., 2005. Genome sequences and structures of two biologically distinct strains of grapevine leafroll-associated virus 2 and sequence analysis. Virus Genes 31, 31–41.

Martin, R.R., Tzanetakis, I.E., Gergerich, R.C., Fernandez, G., Pesic, Z., 2004. Blackberry yellow vein associated virus: a new crinivirus found in blackberry. Acta Hort. 656, 137–142.

Martin, R.R., Tzanetakis, I.E., 2005. Characterization of three novel viruses infecting raspberry. Phytopathology 95, S66.

Mawassi, M., Mietkiewska, E., Gofman, R., Yang, G., Bar-Joseph, M., 1996. Unusual sequence relationships between two isolates of *citrus tristeza virus*. J. Gen. Virol. 77, 2359–2364.

Napuli, A.J., Alzhanova, D.V., Doneanu, C.E., Barofsky, D.F., Koonin, E.V., Dolja, V.V., 2003. The 64-kilodalton capsid protein homolog of *Beet yellows virus* is required for assembly of virion tails. J. Virol. 77, 2377–2384.

Page, R.D.M., 1996. TreeView, An application to display phylogenetic trees on personal computers. Comp. Appl. Biosci. 12, 357–358.

Peng, C.W., Peremyslov, V.V., Mushegian, A.R., Dawson, W.O., Dolja, V.V., 2001. Functional specialization and evolution of leader proteinases in the family *Closteroviridae*. J. Virol. 75, 12153–12160.

Peremyslov, V.V., Pan, Y.W., Dolja, V.V., 2004. Movement protein of a closterovirus is a type III integral transmembrane protein localized to the endoplasmic reticulum. J. Virol. 78, 3704–3709.

Reed, J.C., Kasschau, K.D., Prokhnevsky, A.I., Gopinath, K., Pogue, G.P., Carrington, J.C., Dolja, V.V., 2003. Suppressor of RNA silencing encoded by *Beet yellows virus*. Virology 306, 203–209.

Rott, M.E., Jelkmann, W., 2001. Characterization and detection of several filamentous viruses of cherry: adaptation of an alternative cloning method (DOP-PCR), and modification of an RNA extraction protocol. Eur. J. Plant Path. 107, 411–420.

Rozanov, M.N., Koonin, E.V., Gorbalenya, A.E., 1992. Conservation of the putative methyltransferase domain: a hallmark of the 'Sindbis-like' supergroup of positive-strand RNA viruses. J. Gen. Virol. 73, 2129–2134.

Ruiz-Ruiz, S., Moreno, P., Guerri, J., Ambros, S., 2006. The complete nucleotide sequence of a severe stem pitting isolate of *Citrus tristeza virus* from Spain: comparison with isolates from different origins. Arch. Virol. 151, 387–398.

Sentandreu, V., Castro, J.A., Ayllon, M.A., Rubio, L., Guerri, J., Gonzalez-Candelas, F., Moreno, P., Moya, A., 2006. Evolutionary analysis of genetic variation observed in *citrus tristeza virus* (CTV) after host passage. Arch. Virol. 151, 875–894.

Strik, B., Martin, R.R., 2003. Impact of *Raspberry bushy dwarf virus* on 'Marion' blackberry. Plant Dis. 87, 294–296.

Tairo, F., Musaka, S.B., Jones, R.A.C., Kullaia, A., Rubaihayo, P.R., Valkonen, J.P.T., 2005. Unravelling the genetic diversity of the three main viruses involved in Sweet Potato Virus Disease (SPVD), and its practical implications. Mol. Plant Pathol. 6, 199–211.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tzanetakis, I.E., Martin, R.R., 2004. First report of *Beet pseudo yellows virus* in blackberry in the United States. Plant Dis. 88, 223.

Tzanetakis, I.E., Keller, K.E., Martin, R.R., 2005a. The use of reverse transcriptase for efficient first and second strand cDNA synthesis from single and double-stranded RNA templates. J. Virol. Meth. 124, 73–77.

Tzanetakis, I.E., Postman, J.D., Martin, R.R., 2005b. A member of the *Closteroviridae* from mint with similarities to all three genera of the family. Plant Dis. 89, 654–658.

Tzanetakis, I.E., Postman, J.D., Martin, R.R., 2005c. Characterization of a novel member of the *Closteroviridae* from *Mentha*. Phytopathology 95, 1043–1048.

Tzanetakis, I.E., Suisumathu, J., Gergerich, R.C., Martin, R.R., 2006. Nucleotide sequence of blackberry yellow vein associated virus, a novel member of the *Closteroviridae*. Virus Res. 116, 196–200.

Tzanetakis, I.E., Martin, R.R., 2007. Strawberry chlorotic fleck: identification and characterization of a novel *Closterovirus* associated with the disease. Virus Res. 124, 88–94.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415.